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(54) Title: A METHOD FOR THE SELECTION AND GROWTH OF OOCYTES SUITABLE FOR EMBRYONIC DEVELOPMENT

(57) Abstract: The present invention concerns a method for the culture of oocytes suitable for embryonic development in a synthetic medium containing taurine. The oocytes, coming from female mammals, are pre-selected SN oocytes based on the morphology of the nuclear chromatin, highlighted with Hoechst 33342 fluorochrome dye, matured and fertilized *in vitro*, and then grown, until the blastocyst stage, in a synthetic medium containing taurine.

TITLE

A method for the selection and growth of oocytes suitable for embryonic development.

FIELD OF THE INVENTION

The invention refers to the technical field of *in vitro* cell culture particularly aimed to the optimization of *in vitro* fertilization techniques.

BACKGROUND ART

The *in vitro* culture of oocytes isolated from ovaries is a widely used and well detailed process (Schroeder AC et al. J Exp Zool 1991; 258: 240-245) for different species of mammals. The maturation process of oocytes and the *in vitro* growth during the very early stages of embryo development are nevertheless very critical stages. The success of replantation and subsequent embryo development depends on their correct completion.

The first sign of oocytes maturation *in vitro* is meiosis activation, through the easily recognizable metaphase II stage. After fertilization, the completion of the following development stages, from the 2 or 4-cell embryo stage, until blastocyst stage, thus also represent a sign of the correct maturation of the oocyte. Although all these stages are well defined from a morphological point of view, the same is not true for the definition of the different contribution that various factors, such as the oxygen concentration, the presence of various nutrients or salts in the culture medium, the type of incubator used etc., give, contributing to the overall optimal embryo development. This definition would allow the optimization of *in vitro* oocytes growth protocols, making them usable with improved efficiency at the industrial level also in the zootechnical field.

In the zootechnical field different solutions have been suggested so far, for instance in EP 0387295, which describes a culture medium for oocyte comprising insulin and glucorticoids. A culture medium containing iron-chelating substances is also claimed in GB 2245589 together with a method for the growth of fertilized oocytes. In EP453453 the LIF (Leukemia Inhibitory Factor) is used to improve the *in vitro* development of embryos for reimplantation. However, up to now, *in vitro* oocytes maturation protocols and their following development until the blastocyst stage, gave good reimplantation results only in co-culture with other cell types, which differs according to the mammalian species chosen. Co-culture cells are cumulus cells in mice, especially when used in low oxygen atmosphere conditions (Schroeder AC, Eppig JJ., Dev Biol 1984; 102: 493-497; van de Sandt JJ, Schroeder AC, Eppig JJ., Mol Reprod Dev 1990; 25: 164-171). Other types of co-culture cells have been used in different animal species: for instance oviduct cells in goats (Yadav PS, Saini A, Kumar A, Jain GC. Anim Reprod Sci 1998; 51: 301-306) or "ampulla cells" of the oviduct in man (Hwu YM, Lee RK, Chen CP, Su, JT, Chen YW, Lin SP. Hum Reprod 1998; 13: 1916-1921), or liver cells in cattle (Rehman N, Collins AR, Suh TK, Wright RW., Mol Reprod Dev 1994; 38: 251-38: 251-255) or "granulosa cells" in pigs (Motlik J, Nagai T, Kikuchi K., J Exp Zool 1991; 259: 386-391) and in man (Freeman MR, Whitworth CM, Hill GA., Hum Reprod 1995; 10: 408-414). However, under growth conditions in synthetic media performed without co-cultured cells, the antral oocytes, despite being able to reach meiosis and the metaphase II stage, are fertilized *in vitro* with poor efficiency i.e. in mice (Cecconi S, D'Aurizio R, R. Colonna, J Reprod Fertil 1996; 107: 207-214) and lately, at embryonic level, they develop with poor efficiency both in mice

(Zuccotti M, Giorgi Rossi P, Martinez A, Garagna S, Forabosco A, Redi CA., 1998; Biol Reprod 58: 700-704) as well as in monkeys (Gilchrist RB, Nayudu PL, Hodges JK., Biol Reprod 1997; 56: 238-246).

In vitro culture systems, particularly those optimized to avoid co-culture with other cell types, have the advantage of being generally cheaper, less complex and therefore more easily standardized than the first ones. New formulations able to replace the contribution given by cells in co-culture are therefore highly desirable: for this purpose the identification of essential components in the various growth stages, at first in germinal cells and then in the embryo development is essential. Numerous factors have been studied so far: e.g. it has been shown that variations in the concentration of glucose and inorganic phosphates in the oocyte culture medium, increase the efficiency of embryo cellular division in mice (Scott L, Whittingham DG., Mol Reprod Dev 1996; 43: 336-346) and in hamsters (Schini SA, Bavister BD., Biol Reprod 1998; 39: 1183- 39: 1183-1192), whereas the presence of organic osmolites, namely glutamine, glycine and taurine help maintain a correct cellular homeostasis (Takahashi Y, Kanagawa H., J Vet Med Sci 1998; 60: 433-437).

Taurine is a β-amino acid, used in the culture media as an osmolite (Dumoulin JC, van Wissen LC, Menheere PP, Michiels AH, Geraedts JP, Evers JL, Biol Reprod 1997; 56: 739-744) and antioxidant (Boatman DE., Hum Reprod 1997; 12: 133-149). Furthermore, taurine has been shown to improve the culture efficiency of oocytes (Funahashi H, Kim NH, Stumpf TT, Cantley TC, Day BN, Biol Reprod 1996; 54: 1412-1419) and embryos (C Guyader-Joly, Guerin P, Renard JP, Guillaud J, Ponchon S, Menezo Y., Amino Acids 1998; 15: 27-42) of different

species. However, it is known that, in *in vitro* conditions not all the oocytes are able to complete maturation up to the blastocyst stage. For instance, EP0387295 proposes a selection of suitable and unsuitable oocytes for development, based on the oocyte dimensions (large sized oocytes would prove suitable for development).

The authors of the present invention have earlier suggested that, in the murine model, it is possible to select from the antral oocytes isolated from ovaries, an oocyte population with a better competence to development, on the basis of their chromatin organization (Zuccotti M, Piccinelli A, Giorgi Rossi P, Garagna S, Redi CA, Mol Reprod Dev 1995, 41: 479-485; Zuccotti M, Giorgi Rossi P, Martinez A, Garagna S, Forabosco A, Redi CA, 1998, Biol Reprod 58: 700-704) observed by fluorescence microscopy in the presence of the Hoechst fluorochrome dye 33342 (H bisbenzimide 33342). Under these conditions, oocytes are respectively divided into SN (Surrounded Nucleolus) oocytes, which show a chromatin ring surrounding the nucleolus and a thread-like nuclear chromatin and in NSN (Not Surrounded Nucleolus), without a less defined chromatin surrounding the nucleolus and a more homogeneously nuclear chromatin staining. However, also SN type oocytes, grown *in vitro* according to the known culture methods, are unable, once fertilized, to develop beyond the 4-8-cell embryo stage (Zuccotti M, Giorgi Rossi P, Martinez A, Garagna S, Forabosco A, Redi CA., 1998, Biol Reprod, 58:700-704).

SUMMARY

The present invention provides an efficient method for the maturation and growth of mammalian oocytes in culture in the absence of co-cultured cells, which allows embryo development until the blastocyst stage. The method comprises the

following stages: a) isolation of oocytes from ovarian tissue b) selection of an oocyte population of SN morphology (Surrounded Nucleus), defined on the basis of the nuclear chromatin organization after staining with a supravital dye, such as Hoechst fluorochrome 33342, c) incubation of the SN oocytes until the metaphase II stage, recognized for the presence of metaphasic chromosomes; d) *in vitro* fertilization; e) maturation of the fertilized oocytes in suitable culture media containing taurine.

Oocyte selection in step b) of the process has to be performed immediately after isolation.

The combination of a suitable oocyte population selection based on clear morphological observations, with a simple *in vitro* culture protocol allowing fertilized oocytes to reach the development stage of preplantation, makes the oocyte growth and *in vitro* fertilization process efficient, standardizable and economically advantageous.

DESCRIPTION OF THE FIGURES

Figure 1. Morphology of SN and NSN oocytes at different development stages after staining with Hoechst fluorochrome 33342.

After isolation from ovaries, the antral oocytes (a) were stained with Hoechst 33342 and classified as NSN oocytes (b1) and SN oocytes (b2) based on the organization of their chromatin. After *in vitro* maturation according to protocol B, embryo insemination and culture, as described in Example 5, only the SN oocytes reach the blastocyst stage (e2), while the NSN oocytes stop at the 2-cells embryo stage (c1). A large percentage deriving from SN oocytes develops until the "hatching" stage (f2). Magnification: a, x230; b1, b2, c1, c2, d2, e2, f2, x330.

Figure 2. Histograms of oocyte preplantation development.

The histogram shows the preplantation development percentage, through the 2, 4-cell development stages, until the blastocyst stage, cultivated according to four different protocols A1, A2, A3, B (as described in example 4). Protocol B gives the best yields for antral oocytes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes an *in vitro* method for the culture, fertilization and preplantation development of oocytes, which combines the selection of oocytes with a characteristic morphology after staining with supravital dyes, namely Hoechst fluorochrome 33342 (H bisbenzimide 33342) with defined cell culture growth condition.

The oocytes are isolated from ovarian tissue (step a) of the process) or directly taken from the antral follicles by methods known in the art, i.e. with a thin glass needle under sterile conditions; they may be optionally additionally treated to remove follicular cells with chemical, physical or enzymatic methods and are then placed in a suitable culture medium, such as the media generally used for eukaryotic cell culture, or particularly suited for germinal cells growth. Examples of synthetic culture media used for this purpose are the M2 medium (Sigma no. cat. M5910 or M7167), or the M16 medium (Sigma no. cat. M5910 or M7292), which are commercially available.

The oocytes are therefore separated, according to the morphology and the organization of their nuclear chromatin (step b) of the process) as highlighted after staining with Hoechst fluorochrome 33342 (H bisbenzimide) in SN oocytes (Surrounded Nucleolus) and in NSN oocytes. Other supravital dyes can be used

for the purpose, for instance the SYTO series dyes (Molecular Probes) or any other dye able to stain specifically the DNA or the chromatin of the female gamete. Dyes can be used as such or they may be bound to specific antibodies able to recognize chromatin or any of its components.

According to the method of the present invention, oocyte staining is preferably carried out with the Hoechst fluorochrome 33342 (bisbenzimide H), at a concentration lower than 100 ng/ml; preferably comprised between 30 and 70 ng/ml; a 50 ng/ml in M2 medium concentration is the most preferred, for a time not longer than 10' and at a temperature comprised between 35-37°C. It is known however, that little adjustments of the staining times and of the dye concentrations are routinely attainable by the skilled artisan. Temperatures may also be adjusted within the vitality range of oocytes, or in general of eukaryotic cells. These may be varied slightly according to the oocyte species of origin.

The separation of the oocyte sub-population with the SN morphology is performed after staining with Hoechst fluorochrome, and following a brief illumination with ultraviolet light and observation under an inverted fluorescence microscope. According to the method of the present invention, the exposure time of the oocytes to the ultraviolet light in the presence of the Hoechst dye is preferably not longer than 5 seconds and even more preferably not longer than 3 seconds.

The SN oocytes are recognized and therefore separated from the NSN population, because of the presence of a Hoechst-positive chromatin ring around the nucleolus and a thread-like nuclear chromatin. On the contrary, in NSN oocytes, the ring around the nucleolus is not distinguishable, and the chromatin staining is more homogeneously widespread.

The selected SN oocytes are placed in a culture medium for the maturation stage, corresponding to stage c) of the process. For this purpose, commercially available culture media for eukaryotic cells are used. Examples of media which are especially suited for the growth of eukaryotic cells and are also suited for oocytes, are: α-MEM, Ham's F12, DMEM, TC199, KSOF, and others known to skilled artisan.

Culture media used for oocyte growth are prepared as known to the skilled artisan, as already widely known for the culture of eukaryotic cells: for instance, it is known that some of the reagents are usually added to the culture medium at the moment of use: i.e. L-glutamine at a final concentration of 2 mM, sodium pyruvate at a final concentration comprised between 0.1 and 0.5 mM, and foetal bovine serum or serum from other mammalian species, namely horse, rabbit, man etc. Serum is added because of the presence of growth factors or other protein factors, which can be also added to the medium individually. Growth factors which can be added separately or may be combined with each other, in place of whole serum, are: transferrin, insulin, EGF (Epidermal Growth Factor), PDGF (Platelet Derived Growth Factor), FGF (Fibroblast Growth Factor) BSA, etc. When present, the serum is used at a concentration between 2 and 20% in volume. EDTA may be optionally present in the culture media at a concentration lower than 1 mM.

According to a preferred embodiment of the invention, the medium used for the maturation of the oocytes is α-MEM, supplemented with 2 mM glutamine and with fetal bovine serum at a concentration (vol/vol) of 5%. As an alternative to the serum, or in addition to it, BSA (Bovine Serum Albumin) can be added to the medium. According to a particularly preferred embodiment of the invention, the

culture medium used in step c) of the process additionally comprises the amino acid taurine or its derivatives in effective amounts, intended as a taurine concentration comprised between 1 and 10 mM. A concentration of 5 mM taurine is particularly preferred.

The incubation of the SN oocytes in maturation medium in step c) of the process is performed at 37°C, as for any other eukaryotic cells, under controlled atmospheric conditions, which comprises oocyte growth in a gas mixture with an oxygen percentage not higher than 10%, preferably 5%. According to a preferred embodiment of the invention, the gas mixture, during both the incubation stages c) and e) of the process has the following composition: 5% O_2, 5% CO_2, 90% N_2. Oocyte maturation continues until the metaphase II stage is reached, as recognized by the presence of metaphasic chromosomes and by the expulsion of the second polar globule. Observation is carried out with a stereomicroscope in which the observation dish is kept at the optimal incubation temperature comprised between 35° and 37°C.

The oocytes that have reached the MII stage are placed in contact with male gametes in the next step d) of the process, the *in vitro* fertilization, which is carried out according to techniques known to the skilled artisan. The male gametes may be optionally treated before fertilization in order to improve their motility or the penetration ability into the oocyte for example according to the techniques described in Yanagimachi R.: Mammalian fertilization. In: The physiology of Reproduction, Knobil E. and Neil J. et al. editors, Raven Press, New York, pp135-185, 1988. The male gametes are then capacitated, according to methods known in the art. According to a preferred embodiment, the capacitation of male gametes

takes place in Wt medium (Wt: Whittingham DG. J Reprod Fertil (Suppl) 1971; 14: 7-21), supplemented with BSA (Bovine Serum Albumin) at a concentration comprised between 10 and 100 mg/ml, preferably 30 mg/ml. The oocyte fertilization, stage d) preferably takes place in Wt at a sperm concentration comprised between 1 and 2 $x10^6$ sperms/ml. However, this concentration can be varied according to the mammalian species chosen, and to the spermatozoa vitality, by adjusting it with optional dilution or concentration steps (e.g. by centrifugation of the spermatic liquid) and optimized to give mono-spermatic fertilization.

Alternatively, fertilization can also be carried out by microinjection of the male pronucleus into the female oocyte.

The fertilized oocyte, recognizable as a one cell embryo by the appearance of a second polar globule in the perivitelline space, is transferred (stage e) of the process according to the invention) into a suitable culture medium comprising the aminoacid taurine or its derivatives in effective amounts, until the blastocyst or "hatching" stage is reached. By an effective taurine amount is meant a taurine concentration comprised between 1 and 10 mM. In the method according to the invention a taurine concentration of 5 mM is preferably used.

In the process of the invention, oocyte incubations in the maturation and growth media of the fertilized oocytes, corresponding to steps c) and e) respectively, are carried out under controlled atmospheric conditions, in which the oxygen (O_2) content is not higher than 10% (v/v). The other components of the gas mixture are: nitrogen (N_2) (85-95%) and carbon dioxide (CO_2 5%). Preferably the atmosphere has the following composition: 5% O_2, 5% CO_2 and 90% N_2. The incubation under

controlled atmospheric conditions is preferably carried out in gas-tight modular plexiglass incubator-chambers, such as the Sigma R modular chamber (cat. N° Z11, 905-9), put inside a normal cellular culture incubator, kept at a temperature of 37°C.

Under the preferred atmospheric conditions, which comprises a 5% oxygen concentration, growth is continued up to the 4-cells embryo stage, in commercially available culture medium, preferably containing a low glucose concentration. Low glucose concentration means a concentration of less than 50-100 mg/litre. The culture medium may be replaced, also at this stage (step e) of the process), with other media of slightly different composition, according to the nutritional needs of the oocytes, and is as usually added with the components normally used for culturing eukaryotic cells. In a preferred embodiment of the process, the medium used is the M16 medium, containing 36 mg/litre of glucose (mM16, modified M16 medium) and taurine at 5 mM concentration and added with 5% FCS, 2 mM glutamine and sodium pyruvate at a concentration comprised between 0.1 and 0.5 mM. It is known in the art, that the variation in the glucose concentration, which in this case is reduced with regard to the basal composition of the medium, is withstood by adjustments in the concentration of other molecules, mainly salts, so as to maintain an osmolarity suited to mammalian cell culture, according to methods known in the art.

Afterwards, according to a preferred embodiment of the invention, the embryo is transferred into M16 medium, or similar medium, comprising a useful quantity of taurine or its derivatives and a high glucose concentration, until the blastocyst or "hatching" stage is reached. A high glucose concentration in M16 medium,

corresponds to the basal formulation of M16 (bM16, basal M16 medium), containing at least 1000 mg/litre of glucose and additionally a taurine concentration comprise between 1 and 10 mM, preferably 5 mM. As already highlighted in the description with regard to the oocyte maturation step (corresponding to step c of the process), the M16 medium can be replaced with other equivalent media: this substitution can be functional to the nutritional needs of oocytes from different species. For instance the M16 medium, preferentially used for murine oocytes, can be replaced with TC199 or KSOF medium, more suited, for example, to the growth of bovine oocytes.

According to preferred embodiments of the invention, both the medium used in the incubation corresponding to stage b) ("maturation" medium), as well as that used in the incubation corresponding to stage d) (embryo growth) contain besides the additives normally used for cellular cultures of eukaryotic cells (namely glutamine, sodium pyruvate and FCS), a useful quantity of taurine or its derivatives, preferably between 1 and 10 mM, more preferably 5 mM.

The method, according to the invention, is of particular use in the zootechnical field to optimize the reproduction of mammalian species useful for farming, namely: cattle, sheep, pigs, or in the field of scientific research on murine models or, in general, rodents etc., but can be used with slight variations that mainly concern the culture conditions used, easily attainable by a person skilled in the art, also on primates and superior primates.

A further object of the present invention relates to the use of the process described for farm animal cloning, as far as it provides a method for growing oocytes with a better developmental competence and for selecting the cytoplast best suitable for

embryonic development.

EXPERIMENTAL PART

Reagents

All the reagents were supplied by Sigma Chemical Co. (Milan, Italy), unless otherwise stated.

Culture media

The media for oocyte collection (M2; Sigma), insemination (Wt: Whittingham DG. J Reprod Fertil (Suppl) 1971; 14: 7-21) and the culture of embryos (M16; Sigma) were prepared with high purity ("embryo grade") reagents in ultrapure milliQ water (Millipore, Milan, Italy), according to the protocol provided by the supplier. The oocyte growth medium (α-MEM) was supplied by Sigma (cat. no. M 4526) and added with 0.23 mM of sodium pyruvate, 2 mM L-glutamine and 5% (v/v) fetal bovine serum (HyClone Laboratories, Logan, Utah, USA). The embryo growth medium (M16, Sigma) was added with 2 mM L-glutamine and 0.1 mM EDTA. The pH was adjusted to 7.4. The media were filtered using a 0.22 μm filter before use.

Statistical analyses

The experiments were repeated at least in triplicate. The proportions (relative frequencies or percentages) were compared by two-tailed significance tests based on the normal distribution (Bailey NTJ. "Statistical Methods in Biology" London: Unibooks- English Universities Press; 1959: 33-42). The differences between proportions were expressed according to the z-standardized system (in the form of $z = 1.96$, $p = 0.05$). The counts (absolute frequencies) were analyzed on matrixes using the chi-squared test. Means and standard errors (SEM) were compared with the Student t-test. The differences were considered significant with a p value <

0.05. All the tests were checked with the statistical software (Primit, version 3.03 for MS-DOS; Glantz SA. "Primer of Biostatistic" New York: McGraw Hill Inc; 1992).

Example 1: Oocyte collection and growth

F1 mice were derived from the breeding of C57BL6/N and C3H/HeN (Charles River) and were called B6C3-F1. The females used for the experiments were between 4 and 12 weeks old. The animals were kept under controlled conditions with temperature and humidity kept constant (22°C, 60% humidity) with a photoperiod of 14L:10D. The experiments were carried out in accordance with the European (no. 86/609/CEE) and Italian guidelines (no. 116/ 92, 8/94) for the protection of animals used for scientific research.

A group of 40 females was treated at approximately 10 weeks of age (1800 hours) with 7.5 IU eCG (equine chorionic gonadotropin), by means of intraperitoneal injection, to synchronize the ovarian cycle. Females were sacrificed after 48 hours to collect antral oocytes from the ovaries. As a control, a second group of 37 females similarly treated with eCG, was treated after 48 hours with 7.5 IU hCG (human chorionic gonadotropin), and sacrificed 15 hours after the treatment to collect already ovulated oocytes directly from the oviducts.

The oocytes were isolated from the ovaries of treated females (antral oocytes) or from the oviducts of control females (ovulated oocytes), under stereomicroscope (SMZ-2T, Nikon, Japan) keeping the observation dish at a temperature of approx. 35°C.

The ovulated oocytes were briefly treated with hyaluronidase (Type II, 500 IU/ml) in M2 medium without BSA (37°C, 5 minutes approximately), to remove the

"cumulus" cells. After this treatment, the oocytes were extensively washed with Wt medium and transferred in a drop of fresh Wt medium into a 35x10 mm Petri dish (Corning, Bibby Sterilin, UK), under mineral oil, before insemination.

The antral oocytes were collected by picking them up from ovaries with a sterile thin glass needle and were used to: 1) set up the optimum growth conditions as described in Example 2, after being placed in M2 medium and after having been gently pipetted up and down to remove the cumulus cells, or 2) stained with Hoechst for the NSN or SN morphological selection based on chromatin staining and organization (as described in example 5).

Example 2: *In vitro* oocyte maturation

After isolation, the antral oocytes were washed by three changes in fresh M2 medium and one in α-MEM, before being transferred in a drop (2 μl/oocyte) of α-MEM for the *in vitro* maturation.

Two in vitro maturation protocols were set-up, protocol A and protocol B, as described in table 1. Protocol B differed from protocol A due to the addition of 5 mM of taurine (Sigma, cat. no. T7146) to the α-MEM incubation medium, prepared as described in the materials and methods paragraph.

The incubation of the oocytes, both in protocol A and protocol B, was carried out at 37°C, for 15 hours, in an atmosphere made up of: 5% O_2: 5% CO_2: 90% N_2 in a sealed plexiglass modular incubation chamber (Sigma, cat. no. Z11,905-9), placed in a humidified incubator. The efficiency of protocols A and B in the oocyte maturation process, was assessed at two stages 1): upon reaching the metaphase II stage, and 2) after fertilization, upon reaching the one-cell embryo stage. The frequencies obtained with in vivo ovulated oocytes are also reported, for

comparison purposes. The one cell embryo stage was assessed by observing the appearance of a second polar body in the perivitelline space of the oocyte. The results are reported in table 1.

Table 1. Development efficiency of *in vitro* matured oocytes according to protocol A, protocol B and ovulated in vivo.

Stage	GV oocytes (antral)		Ovulated oocytes
	Protocol A % (n)	Protocol B % (n)	% (n)
GV* Oocyte	100 (530)	100 (469)	
MII** Oocyte	55.1 (292)	55.2 (259)	100 (698)
1-cell***	81.2 (237)	72.8 (205)	83.5 (583)

* GV: Germinal Vescicles oocytes

** MII: metaphase II

*** embryonic development at the 1-cell stage has been calculated by considering the oocytes at metaphase II as 100%.

As can be observed from the data shown in table 1, the progression frequency into meiosis (MII stage) of antral oocytes matured *in vitro* according to protocol A or B, is not different in α-MEM with taurine according to protocol B, or without taurine according to protocol A, when the oocytes are grown in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. In both cases approximately 55% of the oocytes reached the metaphase II stage. The data in table 1, as far as the progression into meiosis is

concerned and measured as reaching the metaphase II stage, thus suggest that the addition of taurine (protocol B) does not have a positive short term effect on *in vitro* oocyte maturation.

Example 3: "*In vitro*" oocyte insemination

The sperm was collected from five month old B6C3-F1 mice and incubated for 60' in 100 µl of Wt medium at a final concentration of $1.5x10^6$ sperms/ml. The oocytes that had reached the metaphase II stage, obtained from *in vitro* maturation or in vivo ovulation, were transferred in the insemination drop and incubated at 37°C in a humidified atmosphere at 5% CO_2. Two hours after insemination, the oocytes were washed in fresh Wt medium to remove the excess spermatozoa and incubated for another hour under the same conditions. The one-cell stage putative embryos, recognized by the appearance of a second polar body, were collected and transferred from Wt medium to M16 (2µl/embryo) and covered with mineral oil.

Example 4: Growth of the fertilized oocytes

Growth of the fertilized oocytes was carried out according to protocols A1, A2, A3 for the oocytes matured *in vitro* according to protocol A and according to protocol B for those matured *in vitro* with the protocol B for *in vitro* maturation (IVM) (see Table 2).

Table 2. Culture protocols (medium and atmospheric composition) for oocyte maturation and embryo development.

Culture Step	**Protocol A**			**Protocol B**
In vitro Maturation (Step c) (IVM)	α-MEM 5% O_2:5% CO_2:90% N_2			α-MEM (+ Taurine) 5% O_2:5% CO_2:90% N_2
	A1	A2	A3	B
Embryo Culture (Step e)	bM16 5% CO_2 in air (20% O_2)	mM16, then bM16 5% CO_2 in air (20% O_2)	mM16 then bM16 5% O_2:5% CO_2:90% N_2	MM16 then bM16 (+Taurine) 5% O_2:5% CO_2:90% N_2

In protocol A1 the embryos were incubated in M16 basal medium (bM16) in an atmosphere made up of 5% CO_2 in air (20% O_2) (growth protocol under basal conditions). In protocols A2, A3 and B, the embryo was incubated according to a two step protocol, in which at first the embryos were placed in modified M16 medium (mM16) containing low glucose (0.20 mM glucose) and low phosphate ions (1.00 mM), and were then transferred into M16 medium at the 4-cell stage, at the original high glucose concentrations (5.56 mM) and phosphate (1.17 mM). In protocol A1, instead the embryos were always grown in the higher glucose concentration. In protocol A2, the modified mM16 medium was used until the 4-cell embryonic stage (reached by 2^{nd} day of culture) and replaced only once with basal M16 medium (bM16) from the 4-cell stage onwards, in atmosphere of 5% CO_2 in air (20% O_2). The A3 protocol was modified, with regard to protocol A2, only by the composition of the atmosphere used in the embryo growth stage, made up of: 5% O_2: 5% CO_2: 90% N_2. Protocol B conditions are the same as protocol A3, except for the addition of taurine (final 5 mM) to both the oocyte maturation medium (α-MEM) as well as to the one used for embryo growth (mM16 and bM16 media).

For all protocols the growth conditions were 37°C in a modular chamber, in a humidified incubator.

In table 3 are shown the results obtained using the different culture protocols of the fertilized oocytes.

Table 3. Oocyte development in culture conditions of four different protocols: A1, A2, A3 and B.

Stage	Protocol*							
	A1		A2		A3		B	
	IVM	Ov	IVM	Ov	IVM	Ov	IVM	Ov
	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)
4-cell	22.0 (32)	67.0 (267)	27.0 (21)	85.0 (144)	64.0 (44)	87.9 (29)	57.0 (160)	82.0 (81)
Blastocyst	3.4 (5)	41.6 (165)	0 (0)	68.6 (116)	18.8 (13)	60.6 (20)	29.3 (76)	56.6 (56)
"Hatching"**	n.d.***	n.d.	n.d.	n.d.	30.8 (4)	50.0 (10)	57.9 (44)	62.5 (35)

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* One cell embryos obtained by culture according to protocol A, were divided into three groups and allowed to grow according to the three different protocols A1, A2 and A3. One cell embryo maturated *in vitro* according to protocol B were grown according to protocol B.

** "Hatching" is calculated by considering the oocytes at the blastocyst stage as being 100%.

*** nd: non detected

The following considerations can be deduced from the data shown in table 3:

1) the low development percentage until the 4-cell stage, obtained with protocol A1 or A2 (22% and 27%, respectively) seems to depend more on the high oxygen concentration (20%) rather than on the high (protocol A1) or low (protocol A2) glucose concentration. In fact, by using protocol A3 (which has the same glucose concentration as in protocol A2 but which has an oxygen concentration reduced from 20% to 5%) rather high yields of 4-cell stage embryos are obtained. The low glucose concentration used in protocol A1, appears to be advantageous for the development of the antral oocytes if associated with a low oxygen concentration (5%) (protocol A2 vs A3: 27% vs 64.0% of development until the 4-cell stage). Results similar to those obtained with protocol A3 at this stage of development (64%), have been obtained with protocol B (57%) which differs only for the presence of taurine (5 mM) into the M16 medium.

2) The presence of taurine does not seem to improve the attainment yields of embryos until the 4-cell stage (protocol A3 vs B).

3) After the 4-cell stage, the presence of taurine becomes important, rendering the difference between protocol A3 (18.8%) and protocol B (29.3%) significant, for the oocytes that reach the blastocyst stage. The positive effect of taurine is even more evident in the next development stage: from blastocysts to the "hatching" stage (as observed comparing the results obtained with protocol B with the ones obtained using protocol A3, 30.8% vs 57.9%; z= 1.591, p= 0.111). In fact, protocol B improves the blastocyst maturation percentage ("hatching") with a total increase of 27.1%. On the contrary, for oocytes ovulated in vivo, the addition of taurine was not particularly advantageous neither in the development stage up to blastocyst

(A3 vs B, 60.6% vs 56.6%), nor in the next "hatching" stage (12.5% total increase from A3 to B, z= 0.711, p= 0.477).

In an additional experiment, the number of cells constituting the embryo at the blastocyst "hatching" stage (96 hours of development) were counted according to the method of Ebert (Ebert KM, Hammer RE, Papaionnau VE. Experientia 1985; 41: 1207-1209). The data obtained showed that the number of cells in the blastocysts obtained from antral oocytes is not statistically different from that of blastocysts derived from *in vivo* ovulated oocytes, when oocytes were grown according to protocol B. The percent mean values were: 32.7 ± 3.6 and 35.3 ± 2.5 obtained respectively in blastocysts derived from antral oocytes and from *in vivo* ovulated oocytes, and are not statistically different from each other (p= 0.562), even when compared with the number of cells in blastocysts developed *in vivo*.

In conclusion, when protocol A1 was used (basal growth conditions), 41.6% of the oocytes ovulated *in vivo* developed until the blastocyst stage, whereas only 3.4% of the antral oocytes matured *in vitro* reached this stage. Instead, when protocol A2 was used, up to 68% of the oocytes ovulated in vivo, and treated as described, develop until the blastocyst stage. However, protocol A2 does not change the development percentage of antral oocytes (0%) matured *in vitro*; such protocols (A1 and A2) thus do not prove suitable for the in vitro maturation of antral oocyte.

When the partial pressure of oxygen was reduced from 20% to 5% (protocol A2 vs A3), the percentage of embryos derived from antral oocytes that reach the blastocyst stage (IVM), goes from 0 to 18.8 (z= 3.695, p= 0.000). Considering the next development stage (the "hatching" stage), the difference between protocol A2 and A3 increases considerably for both the ovulated oocytes (Ov) as well as for

the antral ones matured *in vitro* (IVM) going from zero (n.c.) to 50.0% and from 0 to 30.8%, respectively.

Figure 2 summarises the results obtained with the four maturation and growth protocols for embryos. In particular, protocols A1 and A2 prove unfit for the culture of antral oocytes, in the absence of cumulus cells. In fact, only a very low percentage (3.4%) of oocytes grown under protocol A1 conditions reaches the blastocyst stage (0% with protocol A2). The results instead obtained with protocols A3 and B suggest that, although the presence of taurine during the meiotic progression of the oocyte cycle does not give a particular advantage for the embryo development until the 4-cell stage, its presence in the medium significantly improves the competence to oocyte development beyond this stage. In fact, the embryos reaching the 4-cell stage with protocol B have a better chance of developing until the blastocyst stage.

Example 5: Oocyte selection based on the NSN/SN phenotype and their *in vitro* maturation.

The selection procedure of the isolated antral oocytes as described in example 1, based on the recognition of the presence or not of a chromatin ring around the nucleolus, was carried out according to the procedure described in: Zuccotti M, Piccinelli A, Giorgi Rossi P, Garagna S, Redi CA. Mol Reprod Dev 1995; 41: 479-485, which is herein incorporated as a reference.

The procedure is here briefly described: each isolated oocyte was transferred in a drop (5 µl) of M2 medium containing the Hoechst fluorochrome 33342 (50 ng/ml) and incubated at 37°C for 10 minutes. After staining, the oocytes were classified as belonging to the SN or NSN type, due to the presence or absence, respectively,

of a Hoechst-positive chromatin ring around the nucleolus, when observed under UV light (365 nm excitation; emission 465 nm) with an inverted fluorescence microscope (IX70, Olympus, Japan), 250x magnification, equipped with telecamera (JVC KY-F58). The exposure to UV light was reduced to a minimum (from 3 to 5 secs). Microphotographs were taken using film 640T (Scotch-3M). Figure 1 shows oocytes stained with Hoechst fluorochrome and lit with UV light, having SN (b2) or NSN (b1) morphology, in the various development stages under the culture conditions as defined in protocol B.

The oocytes of the two subpopulations SN and NSN were placed in culture for *in vitro* maturation under protocol B culture conditions. The oocytes that has reached the metaphase II stage, were fertilized *in vitro* as described in Example 3. The results are summarised in table 4. In vivo matured oocytes (ovulated) were used as a control and collected as described in Example 1.

Table 4. Meiotic competence and competence to the development of NSN and SN classified oocytes.

Stage	NSN % (n)	SN % (n)	Ovulated % (n)
GV Oocytes	100 (184)	100 (153)	
MII Oocytes	14.7 (27)	74.5 (114)	100 (99)
1-cell*	70.0 (14)	78.9 (90)	88.9 (88)
4-cell	5.0 (1)	47.4 (54)	81.8 (81)
Blastocyst	0.0 (0)	18.4 (21)	56.6 (56)
"Hatching" **	-	76.2 (16)	62.5 (35)

* the percentage of embryonic development was calculated by considering the number of oocytes at metaphase II as being 100%

** the hatching percentage is calculated by considering the total number of blastocysts as 100%.

Also counted was the number of cells making up the blastocysts at 96 hours of embryo development, resulting in 32.9 ± 2.2 in blastocysts derived from SN oocytes.

As can be seen in table 4, the difference in the competence to development between the NSN and SN antral oocytes, matured *in vitro* according to protocol B, it is indeed apparent in the meiotic maturation stage where only 14.7% of the NSN oocytes compared to 74.5% of SN oocytes reach the metaphase II stage (z= 10.969, p= 0.000). Moreover, the differences obtained upon reaching the blastocyst stage are also highly significative in the two morphologically different subpopolations (0% and 18.4%, respectively; z= 5.880, p= 0.000) (Table 4; Fig. 1). The proportion of blastocysts at the "hatching" stage obtained from the SN population oocytes (76.2%), grown according to protocol B is therefore not significatively different from that obtained with oocytes ovulated in vivo (62.5%) (z= 0.862, p= 0.389), according to a natural process. These data therefore demonstrate the efficiency of protocol B used on SN morphology selected oocytes. On the contrary, the NSN morphology oocytes, even when grown in optimized conditions (protocol B) never developed beyond the 2-cell stage; out of 184 NSN oocytes analyzed, only one reached the 4-cell stage, without going beyond it.

Example 6. Analyses of the fertilization percentages.

The following experiment was performed to gain a better understanding of whether the fertilization rate of antral oocytes (when compared with the fertilization

percentages of ovulated oocytes) depends on changes in the pellucid zone or alterations in the plasma membrane of these oocytes.

Six hours after insemination, the fertilized oocytes were fixed with 2.5% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate (pH 7.4) for 10 minutes at room temperature, then transferred onto a slide and covered with a cover glass. The fertilization and the auto-activation events were measured by the acetic-orcein staining method. The cells were washed with bi-distilled water, fixed with 96% ethanol, stained with 0.5% orcein in 45% acetic acid and washed with 10% acetic acid. The slides were analyzed under a phase microscope at 400x magnification The oocytes were classified as "fertilized" when at least one nucleus or de-condensed pronucleus of sperm with tail was recognizable inside the ooplasm.

The fertilization percentage was measured after oocyte insemination with capacitated spermatozoa, by staining with 0.5% acetic-orcein, 6 hours after insemination, for the presence of decondensed spermatozoa or the formation of more than one pronucleus in the oocyte. The results are shown in table 5.

Table 5. Analyses of the fertilization rate based on the number of pronuclei in the ooplasm

Zona pellucida	Stage	Oocytes	
		IVM* % (n/N)**	Ovulated % (n/N)
Present	Oocyte MII	40.3 (158/392)	7.1 (2/28)
	1 pronucleus	16.6 (65/392)	7.1 (2/28)
	2 pronuclei	31.6 (124/392)	78.7 (22/28)
	>2 pronuclei	11.5 (45/392)	7.1 (2/28)
Removed	Oocyte MII	19.4 (20/103)	6.5 (4/62)
	1 pronucleus	32.0 (33/103)	1.6 (1/62)
	2 pronuclei	24.3 (25/103)	25.8 (16/62)
	>2 pronuclei	24.3 (25/103)	66.1 (41/62)

*IVM: *In vitro* maturation

**n/N: number of oocytes at metaphase II (MII), or containing 1 pronucleus, 2 pronuclei or >2 pronuclei, over the total number of oocytes analyzed in that particular stage.

Table 5 shows that only 31.6% of antral oocytes were fertilized by a single sperm (presence of 2 pronuclei), the remaining being either fertilized by more than one sperm (presence of >2 pronuclei) or auto-activated. Since the blastocysts develop from 1-cell embryos fertilized by a single sperm, the preplantation development efficiency (%) was calculated with respect to this value (31.6%). It follows that the percentage of blastocysts obtained from correctly fertilized antral oocytes was

92.7+15.6% (29.3% out of 31.6%).

This percentage is very similar to that of the *in vivo* ovulated oocytes: 72.0+13.5% (56.6% of blastocysts out of 78.6% of 1-cell embryos), showing the validity of the maturation and fertilization conditions used.

CLAIMS

1) An *in vitro* process for the preplantation growth of mammalian oocytes suitable for embryonic development, which comprises the stages of:

a) oocyte isolation,

b) selection of a Surrounded Nucleolus (SN) morphology oocyte population,

c) incubation of the SN oocytes until the metaphase II stage in a maturation medium,

d) oocyte fertilization,

e) incubation of the fertilized oocytes in a culture medium containing taurine or their derivatives thereof.

2) A process according to claim 1 characterised in that the SN morphology is observed after staining of the oocytes with a supravital dye for chromatin.

3) A process according to claim 2 characterised in that said dye is fluorogenic or is directly or indirectly combined with a fluorochrome.

4) A process according to claim 3 where said fluorogenic dye is the fluorochrome Hoechst N. 33342 (bisbenzimide) and the SN morphology of the oocytes is observed with a fluorescence microscope after illumination with ultraviolet light.

5) A process according to claim 4 characterised in that the Hoechst fluorochrome N. 33342 is used at a concentration of less than 100 ng/ml for a time not longer than 10'.

6) A process according to claim 5 characterised in that said concentration is comprised between 30 and 70 ng/ml.

7) A process according to claim 4 characterised in that said UV illumination is carried out for a time not longer than 5 seconds.

8) A process according to claim 1, wherein steps c) and e) are carried out under controlled atmospheric gas composition, comprising an oxygen (O_2) concentration (vol/vol) not higher than 10% of the total gas mixture.

9) A process according to claim 8 wherein the oxygen (O_2) concentration is lower or equal to 5 % in volume of the gas mixture.

10) A process according to claim 8 wherein the said atmospheric composition is: 5% CO_2, 5% O_2, 90% N_2.

11) A process according to claim 1 wherein the maturation medium in step c) also comprises taurine or its derivatives thereof.

12) A process according to claim 8 characterised in that the fertilized oocyte obtained in step e) of the process is incubated in a low glucose medium until the 4-cells embryo development stage is reached and is therefore transferred to a high glucose medium from the 4-cells development stage onwards.

13) A process according to claim 12 characterised in that the maturation medium in step c) is α-MEM and the culture medium in step e) is M16.

14) A process according to claim 1 wherein the mammal species are chosen from the group consisting of: cattle, sheep, pigs, rodents, primates.

15) A process according to claim 1 wherein the *in vitro* fertilization in step d) occurs by incubation of the oocyte with male gametes or by microinjection of the male gamete into the oocyte pronucleus.

16) A process according to claim 1 wherein in step d) the oocytes are prevoiusly treated either with chemical or enzymatic reagents.

17) A process according to claims 1-16 for use in farm animal cloning techniques

18) Non-human mammalian blastocysts obtainable according to the process of

claims 1-16

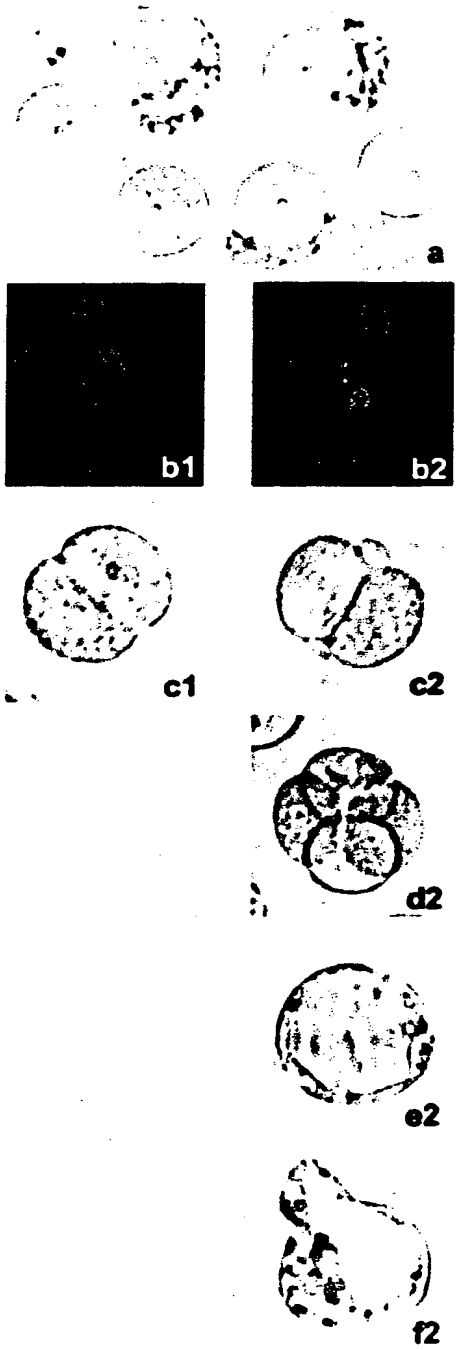

Figure 1

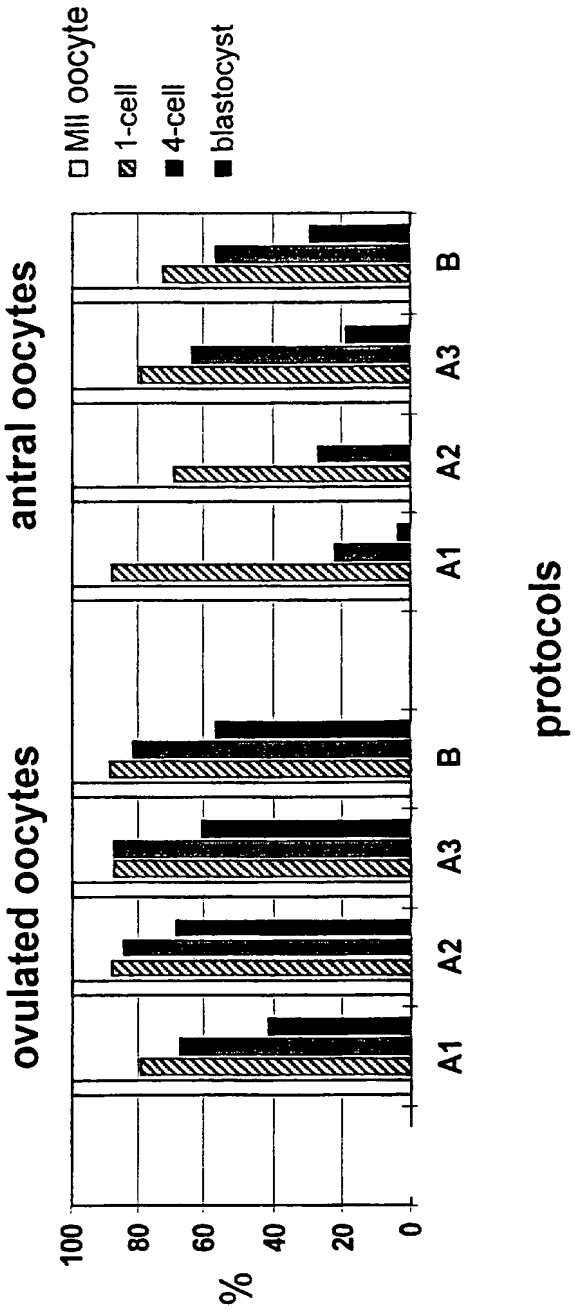
MII oocyte
1-cell
4-cell
blastocyst
ovulated oocytes
antral oocytes
A1
A2
A3
B
A1
A2
A3
B
100
80
60
40
20
0
%
protocols

Figure 2

INTERNATIONAL SEARCH REPORT

Inter[illegible]plication No
PCT/E[illegible]1/02061

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZUCCOTTI M ET AL: "Meiotic and developmental competence of mouse antral oocytes." BIOLOGY OF REPRODUCTION, vol. 58, no. 3, March 1998 (1998-03), pages 700-704, XP001010650 cited in the application the whole document ---	1-17
A	DUMOULIN J C M ET AL: "Positive effect of taurine on preimplantation development of mouse embryos in vitro" JOURNAL OF REPRODUCTION AND FERTILITY, vol. 94, no. 2, 1992, pages 373-380, XP001002585	
X	the whole document ---	18
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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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'A' document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2001	17/08/2001

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHOI YOUNG-HO ET AL: "Effect of taurine on in vitro fertilization and embryo development of BALB/c mouse strain." JOURNAL OF REPRODUCTION AND DEVELOPMENT, vol. 44, no. 1, February 1998 (1998-02), pages 29-34, XP001015534	
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A	TAKAHASHI Y & KANAGAWA H: "Effect of glutamine, glycine, and taurine on the development of in vitro fertilized bovine zygotes in a chemically defined medium." JOURNAL OF VETERINARY MEDICAL SCIENCE, vol. 60, no. 4, April 1998 (1998-04), pages 433-437, XP001015500 cited in the application	
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A	LIU ZISHU & FOOTE R H: "Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent 02." BIOLOGY OF REPRODUCTION, vol. 53, no. 4, 1995, pages 786-790, XP001010651	
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A	LONERGAN P ET AL: "Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension." THERIOGENOLOGY, vol. 51, no. 8, June 1999 (1999-06), pages 1565-1576, XP001010697	
X	the whole document	18

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